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Human Papillomavirus Antibody Responses Among Patients With Incident Cervical Carcinoma

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The human papillomavirus (HPV) is recognized as a major cause of cervical cancer precursor lesions. HPV serology is a key method in the continuing elucidation of the importance of HPV exposure for cancer development and in predicting HPV-associated diseases. To extend previous HPV serological studies on cervical cancer, serum samples from a consecutive series of 216 women with incident untreated cervical carcinoma and 243 age- and sex-matched healthy blood donors were evaluated for the presence of antibodies against HPV capsids, a marker of past or present HPV exposure, as well as against several cervical cancer-associated defined HPV epitopes. Among the capsid antibody responses, HPV type 16 seropositivity had the strongest association with cervical cancer (OR 2.7, 95% CI 1.8–4.2), but HPV 18 and HPV 33 seropositivities were also significantly associated with cervical cancer (OR 1.6, 95% CI 1.1–2.5; and OR 1.5, 95% CI 1.0–2.2, respectively). The antibody responses against the defined HPV epitopes were confirmed to be associated with cervical cancer, at ORs ranging from 1.4 to 2.0. In conclusion, the study confirms that antibodies against defined HPV epitopes are associated with cervical cancer and provides evidence that seropositivities for HPV types 16, 18, and 33 are associated with cervical cancer risk. ***J. Med. Virol. 52:436–440, 1997.***

KEY WORDS: seroepidemiology; ELISA; tumor virology

INTRODUCTION

Infection with the oncogenic HPV types, notably types 16 and 18, is a major cause of cervical intraepithelial neoplasia (CIN) [Schiffman et al., 1993], which can progress to invasive cervical cancer [zur Hausen, 1991]. The study of the antibody response against the oncogenic HPV types is of interest for studies of the epidemiology, immunobiology, and natural history of HPVs, CIN, and cervical cancer.

Serological studies of HPV infections are complicated by the fact that there are many different HPV types, which can cross-react serologically [Dillner, 1995]. However, there are two different approaches that can be used to circumvent this problem. First, intact papillomavirus capsids display a type-specific immunodominant epitope that is destroyed by capsid disruption [Christensen et al., 1990; Kirnbauer et al., 1994]. The use of intact HPV capsids in direct ELISAs, with disrupted capsids as negative controls, has been validated as a type-specific marker of past or present HPV infection [Andersson-Ellström et al., 1996; Chua et al., 1996; J. Dillner et al., 1995a; Heino et al., 1995; Kirnbauer et al., 1994; Wideroff et al., 1995]. HPV capsid serology has been used as a marker of sexual risk-taking behavior [Dillner et al., 1996], to compare the extent of HPV exposure in low-risk and high-risk populations [Nonnenmacher et al., 1995, 1996], and to quantify the excess risk of CIN [Chua et al., 1996] or cervical cancer [Lehtinen et al., 1996] among HPV-exposed women in prospective studies. Second, the use of defined epitopes in serology makes it possible to separate epitopes with serological specificity for HPV infection or HPV-associated diseases from cross-reactive epitopes lacking specificity. Large numbers of seroepidemiological studies have identified a panel of peptide-defined epitopes with serological specificity for HPV infection, CIN, or cervical cancer [Dillner, 1995].

In a previous study of 94 cases of cervical cancer and 188 controls from the Västerbotten county in northern Sweden, seropositivity for HPV type 16 capsids had an odds ratio (OR) for cervical cancer of 9.5, and several

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peptide-defined epitopes also showed strong associations with cervical cancer. A combination of HPV 16 capsid seropositivity and seropositivity for two peptide-defined epitopes, E6:10 and E1:19, showed a particularly strong association [J. Dillner et al., 1995a]. In the present study, we wished to assess the association of HPV serology with cervical cancer, using an independent and considerably larger set of samples. In addition, we investigated whether capsid seropositivities for two oncogenic HPV types (type 18 and 33), for which serological methods have been established more recently, also were associated with cervical cancer.

MATERIALS AND METHODS

Patients and Controls

Serum samples were obtained from 216 women (mean age 53 years, range 23–88 years) with untreated primary invasive cervical carcinoma who were admitted to the Department of Gynecologic Oncology, Radiumhemmet, Karolinska Hospital, Stockholm, Sweden, during 1989–1992. Most tumors were squamous cell carcinomas (SCC; n = 171), and the remainder were adenocarcinomas (ACC; n = 45). The stages of the tumors according to the International Federation of Gynecologists and Obstetricians (FIGO) were stage I (n = 121), stage II (n = 57), stage III (n = 33), and stage IV (n = 5). The differentiation grades were well differentiated (n = 80), moderately diffrentiated (n = 92), poorly differentiated (n = 44), and microinvasive (n = 10) carcinomas. Serum samples were obtained from 243 age- and sex-matched healthy blood donors (mean age 48 years, range 28–80 years) attending the Blood Donor Center of the Karolinska Hospital (n = 149), Huddinge Hospital (n = 58), or Linkoping Hospital (n = 38). The results of serum analyses of these cervical cancer patients and 157 controls using a series of first-generation peptide antigens (different from the ones used in this study) has been reported previously [L. Dillner et al., 1995].

Laboratory Methods

HPV DNA detection was performed using Southern blotting. Fresh cone biopsies were cut into four pieces. Two quadrants were sent for histopathological analysis, and the two intervening quadrants were used for HPV DNA detection. Presence of tumor tissue in the specimen was assumed if the intervening quadrants contained tumor tissue in histopathology. Biopsies were obtained from 47 of the 216 patients (21 patients with stage I, 18 with stage II, 5 with stage III, and 2 with stage IV). The specimens underwent proteinase K digestion, and the DNA was precipitated and dissolved in 50 μl PBS with 5 mM EDTA before Pst/BamH1 restriction enzyme cleavage and agarose gel electrophoresis. After transfer to nitrocellulose, a biotinylated probe mix containing HPV DNA types 6, 11, 16, 18, 31, 33, and 35 (Oncor, Inc., Gaithersburg, MD) was used for the analysis of HPV DNA restriction enzyme cleavage patterns. Hybridization was performed under high-stringency conditions. Thirty-one patients were HPV DNA positive for the following types: HPV16 (n = 21), HPV18 (n = 4), HPV31/33/35 (n = 5), and HPVx (n = 1). These results have been described in detail elsewhere [L. Dillner et al., 1995].

Antibody detection was performed using standard ELISA methods, developed and validated in previous studies [Dillner, 1994]. Peptides were synthesized using tBoc chemistry, as described elsewhere [Dillner, 1990]. The amino acid sequences of the peptides are E1:18, VRYKCGKNRETIEKLLSKLL; E1:19, LSKLLCVSPMCMMIEPPKLR; and E6:10, RWTGRCMSCCRSSRTRRETQL. Peptides were coated overnight at room temperature (RT) onto microtiter plates (Costar, Cambridge. MA) and subsequently blocked with 10% horse serum in phosphate-buffered saline (HS-PBS). Sera were diluted at 1:20, 1:30, or 1:100 in HS-PBS and added to the plates for 2 hr at 37°C. A monoclonal antibody to human IgA (alpha chain) or IgG (gamma chain; Eurodiagnostics, Aapeldorn, The Netherlands) was added and incubated for 90 min at 37°C, followed by a horseradish peroxidase-conjugated antibody to mouse IgG (Southern Biotechnology, Birmingham, AL). The absorbance of the same serum sample reacted with wells coated with PBS only was subtracted. Purified baculovirus-expressed HPV type 18 and 33 capsids containing both the L1 and the L2 capsid proteins were produced as described elsewhere [Volpers et al., 1994]. HPV type 16 capsids were a generous gift from Dr. John T. Schiller, National Cancer Institute, Bethesda, Maryland. The capsids were diluted at 1 μg/ml in cold PBS and incubated on the plates overnight at 4°C. As negative controls, disrupted capsids of BPV were used. Before being diluted and coated, the capsids were disrupted by incubation for 3 hr at room temperature in 0.1 M carbonate buffer, pH 9.6. Analysis was then performed as for peptide ELISAs, except that all incubations were at room temperature.

Data Analysis

Odds ratios were calculated using the exact method of conditional logistic regression. The criterion for assigning seropositivity (cut-off level) was preassigned and was the same as that used in previous studies [J. Dillner et al., 1995; Heino et al., 1995]. The data were also analyzed by Mann-Whitney U test, which is independent of the assigned cut-off.

RESULTS

Antibodies to Intact Capsids

HPV16 seropositivity was associated with an OR of 2.7 for cervical cancer (Table I). Repeated analysis using a higher dilution of the serum samples (1:100) gave similar results (32.9% positive cases, 17.3% positive controls; OR 2.3, CI 1.5–3.6). Presence of IgA to HPV 16 capsids was also associated with cervical cancer but less strongly than presence of IgG to HPV 16 capsids (Table I). Seropositivity of IgG to HPV 33 was also associated with cervical cancer, but not as strongly as HPV 16 seropositivity (Table I). The association was somewhat increased when the analyses were repeated

TABLE I. Seroprevalence of IgA or IgG Against HPV Type 16, 18, or 33 Capsids Among Patients With Cervical Cancer and Controls[a]

Capsids	Percentage positive: Cases (n = 216)	Percentage positive: Controls (n = 243)	OR	95% CI	*P* value (Mann-Whitney)
HPV16, IgA	24.3	15.6	1.73	1.10–2.72	0.0424
HPV16, IgG	37.0	17.7	2.74	1.80–4.16	0.0001
HPV18, IgG	29.2	20.1	1.63	1.08–2.48	0.0044
HPV33, IgG	30.9	23.0	1.49	1.00–2.23	0.0156

[a]All antigens were tested on 459 samples. The OR and 95% CI were calculated using conditional logistic regression. *P* values for association of actual antibody levels with case-control status were calculated using the Mann-Whitney U test.

using a higher dilution of the serum samples (1:100; 28.8% positive cases, 18.5% positive controls; OR 1.8, CI 1.2–2.8). Seropositivity of IgG to HPV 18 capsids was also associated with cervical cancer (Table I).

Comparisons of the HPV capsid reactivity with the HPV DNA type in the tumor showed the expected type specificity, but modest sensitivity of the serological assay. IgA to HPV16 capsids was found among 56% of HPV16 DNA-positive patients, not among patients with other HPV types, and among 16% of controls. IgG to HPV16 was also found among 56% of HPV16 DNA positive patients but also among 40% of patients with other HPV DNA types and among 18% of healthy controls. The overrepresentation of HPV16 IgG seropositivity among patients with infection by other HPV types compared to controls has been a consistent finding in several previous studies [Chua et al., 1996; Dillner et al., 1996; Kirnbauer et al., 1994; Wideroff et al., 1995]. It appears to be due mostly to an overrepresentation of previous HPV16 exposure due to the similar mode of transmission of the anogenital HPVs, because this covariation is reduced by adjusting for sexual history (lifetime number of sexual partners) [Dillner et al., 1996]. Also, for HPV18 and HPV33, 50% of DNA-positive patients were also seropositive for the corresponding HPV capsid types, but the small numbers of patients precluded statistical analysis.

Comparison of seropositivities among patients with squamous cell carcinoma (SCC) or with adenocarcinoma (ACC) showed that HPV16 IgA and HPV33 IgG were three to four times more common among patients with SCC ($P = 0.005$ and 0.008, respectively). HPV16 IgG tended to be more common among SCC patients (OR 1.9, $P = 0.09$), whereas HPV18 seropositivity was equally common among ACC and SCC patients. Comparison of the antibody reactivities with the FIGO stages of the tumors showed no statistically significant differences.

Antibodies Against Peptide-Defined Epitopes

The IgA and IgG antibody responses against three peptide-defined epitopes, designated E1:18, E1:19, and E6:10, previously found to detect a cervical cancer-associated serologic response [J. Dillner et al., 1995a], were chosen for evaluation in this study. Associations with cervical cancer were detected in the dichotomous and/or in the continuous test for all three antigens (Table II).

Three of the serological assays were combined in a way, assigned before the start of the study, that had previously been found to give increased predictive values for cervical cancer [J. Dillner et al., 1995b]. The association with cervical cancer was somewhat increased (Table III).

DISCUSSION

The main novel result of the present study was the finding that seropositivities for HPV types 18 and 33 were also associated with cervical cancer, as had previously been established for HPV type 16 seropositivity. A number of the observations made in the present study, e.g., the association with cervical cancer both of IgA and IgG to HPV 16 capsids and of antibodies to HPV peptide-defined epitopes, the approximately 50% sensitivity of the capsid serology, and the association of HPV 16 and 33 primarily with cervical squamous cell carcinoma, all confirm a number of previous seroepidemiological studies. However, the present study has the advantage that it is a comparatively large study, in which all serum samples were drawn from untreated patients with incident disease. Use of prevalent cases introduces several important measurement and selection biases [Dillner, 1993, 1995]; e.g., the HPV antibody levels are strongly affected by treatment [Dillner, 1993; Elfgren et al., 1996; Lenner et al., 1995]. A further advantage of this study is that it uses assays with documented specificity, validated using serum samples from negative control populations. The HPV capsid serological assays were validated using a panel of serum samples taken from sexually inexperienced women, none of which contained measurable levels of specific antibodies [Andersson-Ellström et al., 1996]. The seroprevalences are linearly increasing with the number of lifetime sexual partners, at about 3–4% per partner, indicating specificity for the sexually transmitted HPV types [Dillner et al., 1996]. Finally, comparisons of seropositivity with the type of HPV DNA found have indicated that the capsid serology is mostly, albeit not exclusively, HPV type specific [Andersson-Ellström et al., 1996; Chua et al., 1996; Dillner et al., 1996; Kirnbauer et al., 1994; Wideroff et al., 1995].

The peptide-based assays used were validated with a

TABLE II. Presence of Antibodies (IgG or IgA) Against HPV-Derived Epitopes Among Cases and Controls[a]

Epitopes	Percentage positive: Cases (n = 216)	Percentage positive: Controls (n = 243)	OR	95% CI	*P* value
E1:18, IgA	45.3	28.8	2.04	1.40–2.98	0.0001
E1:18, IgG	34.2	24.7	1.58	1.07–2.35	0.0013
E1:19, IgA	39.5	32.5	1.36	0.93–1.97	0.0010
E1:19, IgG	34.2	25.9	1.48	1.00–2.19	0.0001
E6:10, IgA	37.9	26.8	1.67	1.14–2.45	0.0001
E6:10, IgG	32.5	25.1	1.44	0.97–2.13	0.0005

[a]The OR and 95% CI were calculated using conditional logistic regression. *P* values for association of actual antibody levels with case-control status were calculated using the Mann-Whitney U test.

TABLE III. Combined Analysis of the Three Serological Assays That Showed the Strongest Association With Cervical Cancer (E1:19 IgG, E6:10 IgA, and HPV16 VLP IgG)[a]

Number of assays positive	Percentage positive: Cases	Percentage positive: Controls	Odds ratio	95% CI	*P* values
One or more	65.0	54.7	1.54	1.07–2.21	0.0210
Two or more	32.5	14.8	2.77	1.78–4.32	0.0000
Three	7.0	0	25.66	4.36–inf.	0.0000
Two assays combined					
E1:19IgG and E6:10IgA	21.3	11.1	2.16	1.29–3.63	0.0033
E1:19IgG and HPV16 IgG[b]	12.5	3.3	4.20	1.86–9.45	0.0005
E6:10IgA and HPV16 IgG[b]	16.2	2.5	7.64	3.15–18.6	0.0000

[a]All 459 human serum samples were analyzed. The OR, 95% CI, and *P* values were calculated using conditional logistic regression.
[b]Dilution of serum sample 1:100.

panel of adult women repeatedly testing negative by general primer PCR for HPV DNA [Strickler et al., 1997]. In longitudinal studies, seroconversions against both capsid and peptide antigens occur simultaneously [Wikström et al., 1995]. However, comparisons with the infecting type of HPV DNA indicate that the peptide-defined epitopes have less HPV type specificity [Wikström et al., 1995].

The comparisons of the IgG and IgA reactivities found only minor differences. There were two rationales for comparing these isotypes. IgG antibodies are in general more stably persisting after viral clearance, and the IgA levels in serum are correlated with the IgA in cervical secretions [L. Dillner et al., 1989]. In theory, IgA should therefore primarily measure presently active mucosal infections. However, although some studies of CIN found higher odds ratios for IgA than IgG [J. Dillner et al., 1989; Strickler et al., 1997], the Ig isotype reactivity has been found to be variable for different antigens [Dillner et al., 1994; J. Dillner et al., 1995a; Strickler et al., 1997]. In this study, the only notable difference between the IgA and IgG responses was the better HPV type specificity of the HPV 16 capsid IgA response.

Compared to our previous study of cervical cancer cases from the Västerbotten county in northern Sweden, higher proportions of healthy controls were seropositive, in both the HPV capsid and the HPV peptide-based assays. The lowest HPV16 capsid seroprevalences among healthy women (2%) have been found in a population-based serum sample bank collected in Finland in 1966–1970 [J. Dillner et al., 1995b; Lehtinen et al., 1996]. A sample of healthy blood donors from the same hospital as in this study, but with 15 years higher mean age, had a seroprevalence of only 4% [Heino et al., 1995a]. By contrast, several studies of contemporary young Scandinavian women have found HPV seroprevalences of 15–16% [Chua et al., 1996; Olsen et al., 1996], which is more similar to the seroprevalences of 18–23% found in this study.

The seroprevalences of both herpes simplex type 2 [Forsgren et al., 1994] and of *Chlamydia trachomatis* [Persson et al., 1995] have shown pronounced increases from the 1960s to the 1980s in Sweden, in parallel with a changing life style, with an increased number of relationships before permanent cohabitation/marriage. The different HPV seroprevalences in different populations parallel the seroprevalences of other sexually transmitted diseases (STDs) [Nonnenmacher et al., 1995, 1996]. HPV serology would therefore appear to have its strongest predictive value for HPV-associated diseases in populations with a low STD prevalence. The HPV serology-associated odds ratio for cervical cancer will thus vary depending on the population studied, with the highest odds ratios found in populations with low STD risk and low cervical cancer incidence, such as in our previous studies from Västerbotten in northern Sweden [Dillner et al., 1994; J. Dillner et al., 1995a] or from Finland in the 1960s [Lehtinen et al., 1996].

In contemporary Scandinavia, HPV serology appears to be useful mainly as an epidemiological marker for

sexual behavior in populations. Ecological studies comparing HPV seroprevalences in different regions and over time to provide a better understanding of the epidemiology of the infection could both shed new light on the role of HPV in cancer [Dillner, 1995] and allow design of appropriate preventive actions [Dillner, 1997].

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